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TOPIC REVIEW

Recent advances in the molecular understanding of glioblastoma

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Abstract Glioblastoma is the most common and most aggressive primary brain tumor. Despite maximum treatment, patients only have a median survival time of 15 months, because of the tumor's resistance to current therapeutic approaches. Thus far, methylation of the *O*⁶-methylguanine-DNA methyltransferase (MGMT) promoter has been the only confirmed molecular predictive factor in glioblastoma. Novel “genome-wide” techniques have identified additional important molecular alterations as mutations in isocitrate dehydrogenase 1 (*IDH1*) and its prognostic importance. This review summarizes findings and techniques of genetic, epigenetic, transcriptional, and proteomic studies of glioblastoma. It provides the clinician with an up-to-date overview of current identified molecular alterations that should ultimately lead to new therapeutic targets and more individualized treatment approaches in glioblastoma.

Keywords Glioblastoma · Molecular · (Epi)genetic · Transcriptional · Proteomic

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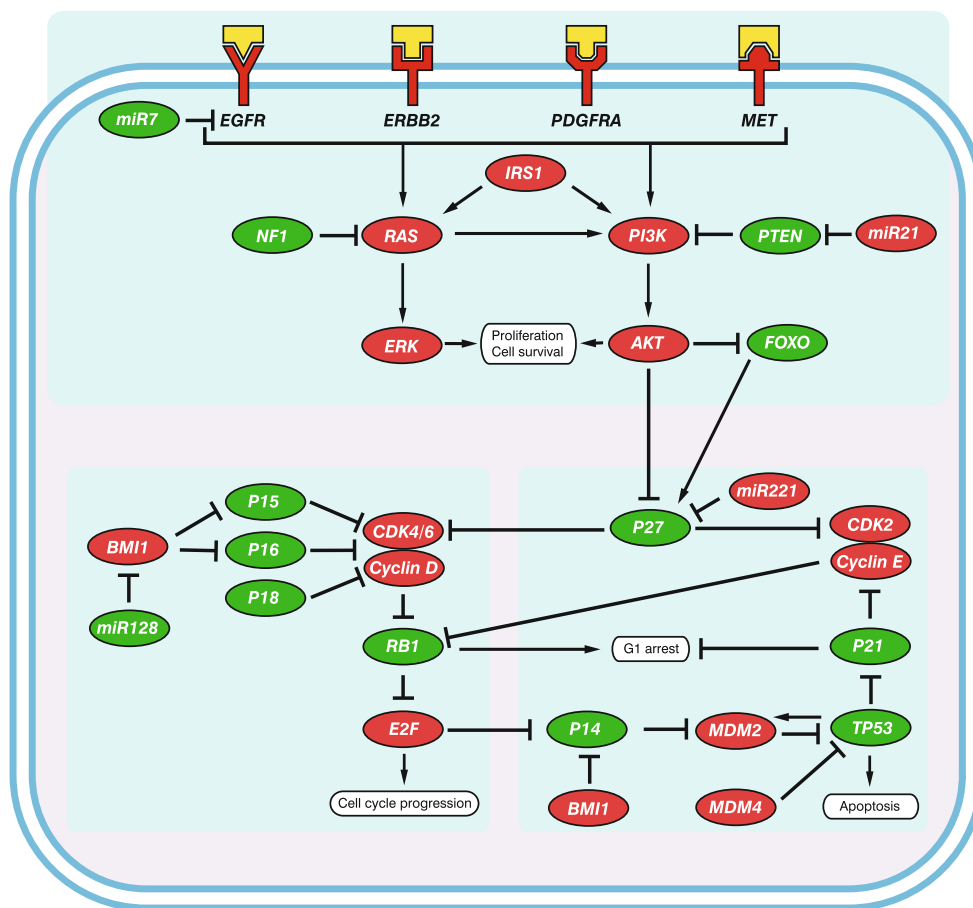
Introduction

Glioblastoma, or astrocytoma WHO grade IV, is the most fatal primary brain cancer found in humans. Most glioblastomas manifest rapidly de novo, without recognizable precursor lesions. These primary glioblastomas present in elderly patients with a brief clinical history and are characterized by rapid progression and short survival time. A small group of young patients has a history of epilepsy caused by low-grade gliomas which, within years, progress to secondary glioblastoma. A secondary glioblastoma occurs in ~5% of glioblastoma patients, and can only be diagnosed with clinical (neuroimaging) or histological evidence of its evolution from a less malignant glioma [1].

The standard treatment for newly diagnosed glioblastoma patients is gross total removal, if possible, followed by the combination of the alkylating cytostatic drug temozolomide (TMZ) and RT [2, 3]. Median overall survival is 15 months only [3], although for a rare group of long-term survivors (2–5%) survival time exceeds 3 years [4, 5]. Differences between patients and their performance status lead to variation in survival, which can be calculated for individual patients by means of nomograms [6]. A better prognosis is associated with younger age, better performance status, and more extensive surgical resection followed by TMZ and RT [6]. In contrast with many other malignancies, however, there have only been small improvements in the glioblastoma patient's prognosis over recent decades. Nevertheless, understanding of the molecular alterations in signaling pathways and the consequent pathology in glioblastoma has greatly increased in recent years and is beginning to match that of other types of cancer.

This review provides an overview of the molecular alterations in glioblastoma (Fig. 1) [7–9]. They are

Fig. 1 Simplified representation and integration of three commonly altered pathways involved in glioblastoma. *Upper panel*, the growth factor receptor/PI3K/AKT pathway. The *lower panels* depict the RB pathway (left) and the P53 pathway (right). Proteins that potentially act as tumor suppressors are indicated in *green* whereas oncoproteins are indicated in *red*. The growth factors binding to the receptors have been depicted in *yellow*



grouped according to the different mechanisms that underlie transformation to the neoplastic phenotype, starting from (epi)genetic, via transcriptional, to proteomic studies of glioblastoma. The important molecular alterations, which have been identified by novel “genome-wide” techniques, are discussed in relation to gliomagenesis and glioma progression and in relation to clinical subgroups and prognosis. Finally, we discuss the application of these new insights in the light of future prospects for experimental and clinical practice in neuro-oncology.

Genomic and genetic variants

Genomic instability

Genomic instability is one of the enabling characteristics of cancer [10]. It can be broadly differentiated into chromosome instability (CIN) and microsatellite instability (MIN or MSI). Cytogenetic studies of glioblastoma have shown that most tumors are near-diploid, and that numerical and structural chromosomal abnormalities are common [11]. MSI is rarely observed for non-inherited newly diagnosed glioblastomas, because of inactivation of mismatch repair

(MMR) genes [12]. However, in recurrent glioblastomas after TMZ treatment, inactivating mutations have been observed in *MSH6*, one of the MMR genes. *MSH6* mutations have not been associated with detectable MSI as manifested by changes in the length of microsatellite sequences, but with a hypermutator phenotype [7, 9, 13]. As genetic alterations and genomic instability are closely linked with each other, it is an interesting finding that in glioblastoma, tumors from short-term survivors have more genetic alterations than long-term survivors’ tumors [5].

Chromosomal alterations

Techniques

Evolving techniques have identified increasingly more detailed chromosomal alterations.

Karyograms [11], fluorescent in situ hybridization (FISH) analyses [14], and comparative genomic hybridization (CGH) [15, 16] have preceded whole-genome single nucleotide polymorphism (SNP)-based arrays. Whereas karyograms are able to reveal only gross chromosomal changes, SNP-based arrays have the ability to detect copy number alterations (CNAs), varying from complete

chromosomal changes to small intragenic deletions. In addition, it is possible to distinguish signals from individual alleles and therefore reveal copy-number-neutral (CNN) loss of heterozygosity (LOH). Here, a chromosome segment is lost, whereas the corresponding homologous region is duplicated, resulting in a neutral copy number. For example, 17p, which contains *TP53*, is a significant region of CNN LOH in glioblastoma [7, 8].

Among chromosomal alterations, amplifications and deletions can be distinguished. Of these, the most common in glioblastoma will be discussed here. Reports of incidental translocations are rare in glioblastoma [17]; consequently, translocations may not be important in the development of glioblastoma and will not be discussed further.

Amplifications

Amplification of the epidermal growth factor receptor (*EGFR*) gene is a characteristic finding in primary glioblastoma (Table 1) [5, 8, 16, 18]. Focal (restricted to a few Mb) and broader (from several Mbs to whole chromosomes) CNAs that include the *EGFR* gene may have different molecular consequences [16]. Focal amplification of *EGFR* correlates with EGFR overexpression or mutations and deletions in the *EGFR* gene, and subsequent activation of the PI3K/AKT pathway [16, 19]. Upregulated PI3K/AKT signaling has been associated with a poor prognosis [20, 21]. Amplification of the complete chromosome 7, containing *EGFR*, *MET* [7], and its ligand *HGF*, has been found to correlate with activation of the MET axis [16]. Furthermore, *EGFR* amplification is reported to appear as double minutes (small fragments of extrachromosomal DNA), and extra copies of *EGFR* have also been found inserted into different loci on chromosome 7 [22]. Remarkably, gain of chromosome 7 and amplification of *EGFR* have been found more frequently in short-term survivors [4, 5], however *EGFR* alterations are not of prognostic importance in glioblastoma [4, 18, 23].

Amplification of 12q13–15, where the oncogenes *CDK4* and *MDM2* are located, results in the disruption of both the RB and P53 pathways [7, 8, 16, 24]. The genes encoding the receptor tyrosine kinases *KIT*, *KDR*, and *PDGFRA*, adjacently located on chromosome 4q12, are frequently found to be (co)amplified [25]. Other amplified regions containing oncogenes, for example *AKT3* [7, 26] and *CCND2* [7, 16], are listed in Table 1.

Deletions

LOH of chromosome 10q is the most common genomic alteration found in both primary and secondary glioblastomas [18, 24] (Table 1) and is associated with poor

survival [5, 18]. Different regions are frequently lost at chromosome 10, including the regions containing *PTEN*, *MGMT* [1, 18], and *ANXA7*, an EGFR inhibitor [27]. Another frequently deleted inhibitor of EGFR signaling is *NFKBIA*, which is located on chromosome 14; this deletion is associated with poor survival [28]. Furthermore, loss of chromosome 9p, which contains a variety of tumor-suppressor genes, including *CDKN2A*, *CDKN2B*, and *PTPRD*, is frequently seen [8, 18, 29], especially in short-term survivors [4, 5]. *CDKN2A* and *CDKN2B* encode three important cell cycle proteins, p14^{ARF} and p16^{INK4A}, and p15^{INK4B} [5, 8, 15, 16, 18], which are involved in the RB and P53 pathways. Deletion of *CDKN2A* and *CDKN2B* is often accompanied by deletion of *CDKN2C* on chromosome 1p32, which encodes another cell cycle protein p18^{INK4C} [15]. LOH of chromosome 1p is found in both primary and secondary glioblastomas [30]. Longstanding speculation about the potentially located tumor suppressor gene at 1p has recently been advanced by identification of the suggested candidate genes *CIC* and *FUPB1* [31]. Co-deletion of 1p and 19q is frequently seen in oligodendrogliomas and is, in those, associated with prolonged survival [4] and translocations [32]. Although this co-deletion has been observed in glioblastomas, no similar association has been identified. Isolated LOH 19q, however, is frequently observed in secondary glioblastoma [5, 30] and may be a marker of longer survival [5].

Somatic mutations

Techniques

In addition to amplifications and deletions, genes implicated in glioblastoma can be affected by somatic mutations. Mutation analysis has identified mutations activating oncogenes and others inactivating tumor-suppressor genes in glioblastoma [7, 9, 33]. The recommended method used to be direct or Sanger sequencing after amplification of the suspected locus by means of polymerase chain reaction (PCR). Nowadays, improved sequencing techniques are being developed and rapidly applied to facilitate genome-wide mutation analysis [34].

Mutations frequently found in glioblastoma

Mutations in “common” cancer genes, for example *TP53* and *PTEN*, are very frequent in glioblastomas, but are not of prognostic importance (Table 2) [4, 7, 9, 18, 23, 33]. Furthermore, glioblastoma-specific mutations are seen; the *EGFRvIII* mutant lacks 267 amino acids in the extracellular part, resulting in a constitutively activated receptor that no longer requires its ligand EGF to signal downstream [35]. *EGFR* point mutations have also been identified in

Table 1 Frequently identified copy number alterations in glioblastoma

Chromosome	Cytoband	Alteration	Frequency (%)	Gene symbol	Gene name	Function of encoded protein	Refs.
1	1p32	Deletion	3–16	CDKN2C	Cyclin-dependent kinase inhibitor 2C	Regulator of cell cycle	[7, 24]
1	1p36	Deletion	14–40	? ^a			[8, 26, 30]
1	1q32	Amplification	4–15	MDM4	Mdm4 p53 binding protein homolog	Apoptosis	[7, 8, 16, 24]
1	1q44	Amplification	2–11	AKT3	V-akt murine thymoma viral oncogene homolog 3	Regulator of cell signaling, involved in cell proliferation and survival	[7, 24, 26]
2	2q22	Deletion	7	LRP1B	LRP1B low density lipoprotein receptor-related protein 1B	Regulator of cell signaling, involved in cell proliferation and survival	[8]
3	3q26	Amplification	0–16	PIK3CA	Phosphoinositide-3-kinase, catalytic, alpha polypeptide	Regulator of cell signaling, involved in cell proliferation and survival	[7, 9, 16]
4	4q12	Amplification	15	KIT	V-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog	Regulator of cell signaling, involved in cell proliferation and survival	[25]
4	4q12	Amplification	15	KDR	Kinase insert domain receptor	Angiogenesis, vasculogenesis and endothelial cell growth	[25]
4	4q12	Amplification	2–18	PDGFRA	Platelet-derived growth factor receptor, alpha polypeptide	Regulator of cell signaling, involved in cell proliferation and survival	[7, 8, 16, 24, 25]
6	6q26-27	Deletion	25	PARK2	Parkinson protein 2	Regulator in targeting proteins for proteasomal degradation	[8]
7	7p11	Amplification	23–66	EGFR	Epidermal growth factor receptor	Regulator of cell signaling, involved in cell proliferation and survival	[7–9, 16, 18, 24]
7	7q21	Amplification	1	CDK6	Cyclin-dependent kinase 6	Regulator of cell cycle	[7]
7	7q31	Amplification	3–19	MET	Met proto-oncogene	Regulator of cell signaling, involved in cell proliferation and survival	[7, 16, 24]
9	9p21	Deletion	26–66	CDKN2A	Cyclin-dependent kinase inhibitor 2A	Regulator of cell cycle	[7–9, 18, 24]
9	9p21	Deletion	31–66	CDKN2B	Cyclin-dependent kinase inhibitor 2B	Regulator of cell cycle	[7, 8, 24]
9	9p23	Deletion	14–46	PTPRD	Protein tyrosine phosphatase, receptor type, D	Regulator of cell signaling, involved in cell proliferation and survival	[24]
10	10q23-24	Deletion	5–70	PTEN	Phosphatase and tensin homolog	Regulator of cell signaling, involved in cell proliferation and survival	[7–9, 16, 18, 24]
12	12p13	Amplification	2–14	CCND2	Cyclin D2	Regulator of cell cycle	[7, 16]
12	12q14	Amplification	7–24	CDK4	Cyclin-dependent kinase 4	Regulator of cell cycle	[7–9, 16, 24]
12	12q14-15	Amplification	7–22	MDM2	Mdm2 p53 binding protein homolog	Apoptosis	[7, 8, 16, 24]
13	13q14	Deletion	3–47	RB1	Retinoblastoma 1	Regulator of cell cycle	[7–9, 16, 30]
17	17p13	Deletion	1–22	TP53	Tumor protein p53	Apoptosis	[7–9, 16]
17	17q11	Deletion	0–11	NF1	Neurofibromin 1	Regulator of cell signaling, involved in cell proliferation and survival	[7, 9]
19	19q	Deletion	11–35	?			[16, 24, 30]
22	22q12.3	Deletion	53	TIMP3	TIMP metalloproteinase inhibitor 3	Extracellular matrix	[77]

A deletion can indicate either a CNV-LOH, an LOH, or a homozygous deletion; a ? indicates that the gene of interest has not yet been identified

^a Genes within the region: CAMTA1, PER3, TNFSF9, VAMP3, PARK7, MIG6, RERE, GPR157, H6PD [8]

Table 2 Genes frequently found to be mutated in glioblastoma

Gene symbol	Gene name	Function of encoded protein	Point mutation (%)	Refs.
EGFR	Epidermal growth factor receptor	Regulator of cell signaling, involved in cell proliferation and survival	14–15	[7, 9, 36]
ERBB2	V-erb-b2 erythroblastic leukemia viral oncogene homolog 2	Regulator of cell signaling, involved in cell proliferation and survival	0–7	[7, 9]
IDH1	Isocitrate dehydrogenase 1 (NADP+)	NADPH production	12–20	[9, 39–42, 44]
NF1	Neurofibromin 1	Regulator of cell signaling, involved in cell proliferation and survival	15–17	[7, 9]
PIK3CA	Phosphoinositide-3-kinase, catalytic, alpha polypeptide	Regulator of cell signaling, involved in cell proliferation and survival	7–10	[7, 9]
PIK3R1	Phosphoinositide-3-kinase, regulatory subunit 1 (alpha)	Regulator of cell signaling, involved in cell proliferation and survival	7–8	[7, 9]
PTEN	Phosphatase and tensin homolog	Regulator of cell signaling, involved in cell proliferation and survival	24–37	[7, 9, 18]
PTPRD	Protein tyrosine phosphatase, receptor type, D	Regulator of cell signaling, involved in cell proliferation and survival	0–6	[9]
RB1	Retinoblastoma 1	Regulator of cell cycle	8–13	[7, 9]
TP53	Tumor protein p53	Apoptosis	31–38	[7, 9, 18]

glioblastoma, in the extracellular domain, whereas they are predominantly found in the kinase domain in other tumor types, for example lung cancer [36]. Two extensive mutational studies have provided an overview of the most common mutations affecting glioblastoma (Table 2) [7, 9]. Although mutations in “common” cancer genes, for example *BRAF* and the *RAS* genes, have rarely been observed in gliomas (<5%) [37], inactivating mutations and deletions have been identified in their inhibitory tumor suppressor gene *NF1* [7]. Mutations in *PIK3CA* and *PIK3R1*, coding, respectively, for the PI3K catalytic subunit p110 α and regulatory subunit P85 α , have been described [7, 9].

The incidence of mutation in glioblastoma is lower than in other solid tumors [38], with the exception of the hypermutator phenotype [13], which, as described above, is found in recurrent glioblastomas after treatment with alkylating agents. This may be caused by *MGMT* methylation or mutational inactivation of DNA-repair enzymes, for example *MSH6* [7, 9, 13].

IDH1 mutations

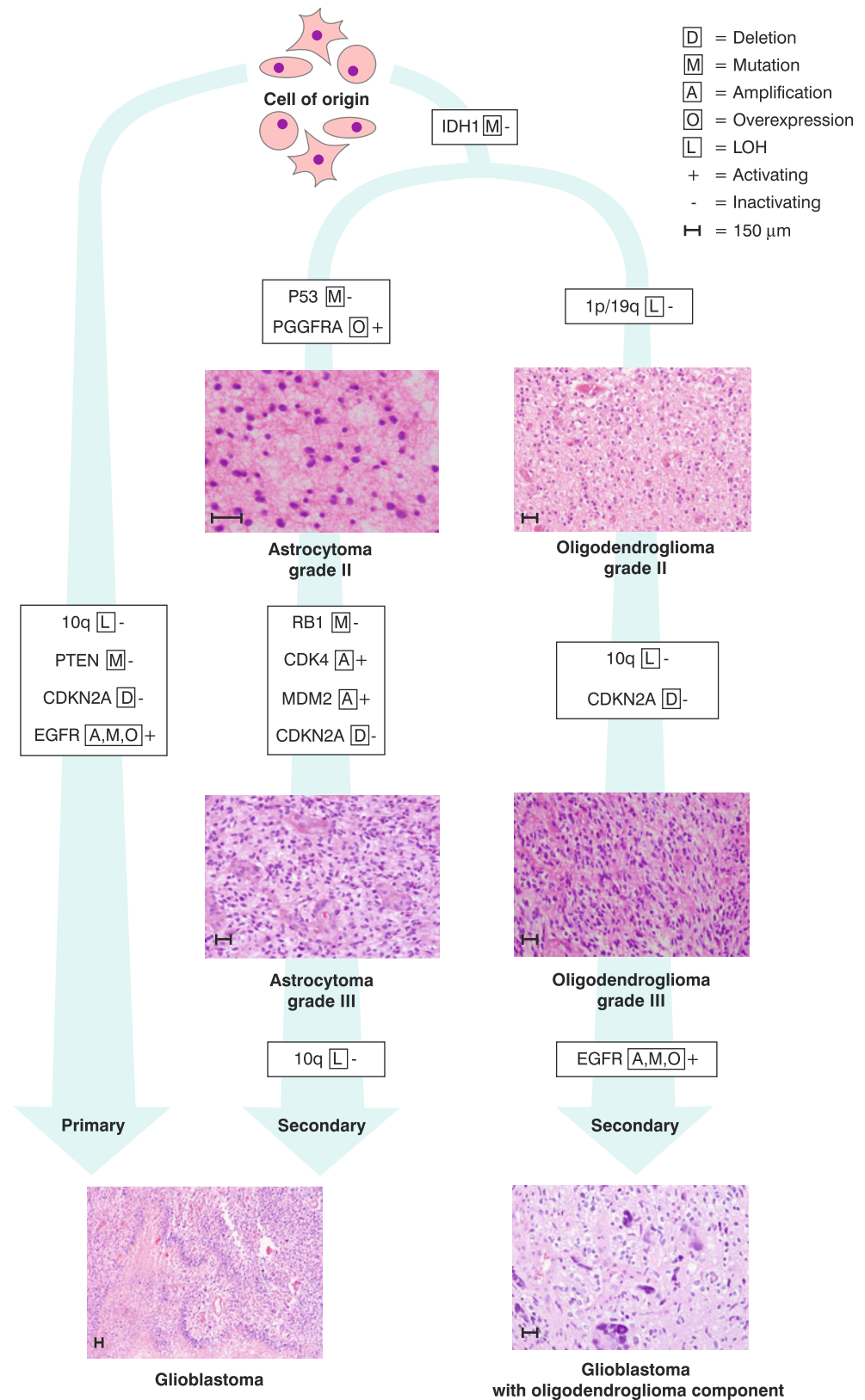
An interesting gene found to contain mutations in glioblastoma is *IDH1*, which encodes isocitrate dehydrogenase 1 and is involved in energy metabolism [9]. *IDH1* mutations have been predominantly identified in secondary glioblastomas and low-grade gliomas, with mutations in more than 70% of cases [9, 39–43]; they are found only sporadically in primary glioblastomas [9, 41–44]. Because patients with *IDH1* mutated primary glioblastomas are generally younger and have longer median survival and

wild-type *EGFR*, which are characteristics of secondary glioblastomas, it is hypothesized that these are in fact secondary glioblastomas for which no histological evidence of evolution from a less malignant glioma is found. Therefore, *IDH1* could be used to differentiate primary from secondary glioblastomas [41]. In different glioblastoma studies *IDH1* mutations have been found to be an independent positive prognostic marker [9, 40, 44, 45]. *IDH1* mutations have been shown to inactivate the enzyme with subsequent HIF-1 α induction [42, 44, 46]. In addition, the mutations result in gain of function to catalyze α -ketoglutarate (α -KG) to 2-hydroxyglutarate (2-HG) [47]. Furthermore, 2-HG inhibits histone demethylases and TET 5-methylcytosine hydroxylases. These α -KG dependent dioxygenases are thought to be involved in epigenetic control. This suggests that mutations in *IDH1* change the expression of a potentially large number of genes [48]. Given that mutations in *IDH1* are an early event in gliomagenesis (Fig. 2) [49], this may implicate widespread alteration of epigenetic control as the key mechanism in gliomagenesis in *IDH1* mutated tumors. Furthermore, it might explain the extensive and fundamental differences between mutated and wildtype *IDH1* glioblastoma.

Polymorphisms

Family members of glioma patients are more susceptible to glioma and other cancer types [50], suggesting a genetic origin. The most common type of genetic variation is formed by single nucleotide polymorphisms (SNPs). A SNP is a single base-pair alteration at a specific locus. They can be identified by PCR for single loci or use of

Fig. 2 Genetic pathways toward primary and secondary glioblastoma



SNP-based arrays for whole genome alterations. SNPs have been linked to susceptibility to glioblastomas. In particular, allergies and asthma's inverse association with

glioblastoma have been observed in different studies and have been linked with polymorphisms in HLA and interleukins. This may suggest that immune factors play a role

in gliomagenesis [51]. SNP309 in *MDM2* has been associated with an increased risk of various types of cancer, but has not been associated as a risk or prognostic factor in respect of glioblastoma in large studies [52]. SNPs in *CDKN2B*, *TERT*, and *RTEL1* have been described in independent studies as susceptibility loci for high-grade glioma [53, 54]. In a follow-up study, SNPs in DNA double-strand break repair enzymes, for example *RTEL1*, have been found to correlate with glioblastoma survival [55]. Various other SNPs have been correlated with glioblastoma survival and age of onset [55], however, these studies' findings have not yet been confirmed.

Gene expression profiling

Techniques and results

Overexpression or underexpression of genes in glioblastoma compared with that in a normal brain or in low-grade gliomas may be an indication of genes that are involved in gliomagenesis (Table 3). Most of the 20,000–25,000 genes encoded by the human genome are known [56], and these have been applied to chips used for micro-arrays. Differences in expression of “unknown” genes can be studied by serial analysis of gene expression (SAGE), by use of small expression tags [57]. Large-scale expression studies are usually validated by reverse transcription (RT)-PCR for individual genes.

A high level of expression of insulin-like growth factor binding proteins, for example IGFBP-2/3 [58], angiogenic factors, for example vascular endothelial growth

factor A (VEGFA) [59], and mesenchymal markers, for example YKL-40/CHI3L1, are frequently seen in glioblastoma (Table 3) and have been associated with poor prognosis [60–62]. In contrast, NOTCH signaling genes, for example DLL3, are indicative of better survival [63]. Furthermore, WEE1, a kinase that regulates the G₂ checkpoint in glioblastoma cells, is commonly overexpressed in glioblastoma and higher expression has been shown to correlate with worse patient survival [64].

Gene expression profiling studies outperform histology for grading and prognosis

Low-grade astrocytomas have rather specific and consistent expression profiles, whereas for primary glioblastomas there is much larger variation between tumors. Furthermore, secondary glioblastomas have distinct expression profiles and features of the other two types [65]. Expression profiling of different types and grades of glioma has been found to outperform histopathologic grading for prognosis [20, 66–68]. To improve classification of patients with glioblastoma, a gene dosage expression incorporated model based on seven genes (*POLD2*, *CYCS*, *MYC*, *AKR1C3*, *YME1L1*, *ANXA7*, and *PDCD4*) has been generated. This model can be used to categorize patients in risk groups with different prognosis; a high-risk group in which ≥ 5 of 7 genes are altered, a moderate-risk (3–4 genes), or a low-risk group (≤ 2 genes). In this study, *MGMT* methylation and *IDH1* mutational status were not incorporated [69]. A newer predictive model based on expression of four genes (*CHAF1B*, *PDLIM4*, *EDNRB*, and *HJURP*) has been generated, and is independent of *MGMT* methylation and

Table 3 Genes frequently found to be overexpressed in glioblastoma compared with either normal brain tissue or low-grade gliomas

Gene symbol	Gene name	Function of encoded protein	Refs.
CD44	CD44 molecule	Cell-cell interactions, cell adhesion and migration	[20, 62]
DLL3	Delta-like 3	Notch signaling	[20, 62]
EGFR	Epidermal growth factor receptor	Regulator of cell signaling, involved in cell proliferation and survival	[62]
FABP7	Fatty acid binding protein 7	Fatty acid uptake, transport, and metabolism	[62]
IGFBP2	Insulin-like growth factor binding protein 2	Regulation of cell growth	[58–60, 62]
IGFBP3	Insulin-like growth factor binding protein 3	Regulation of cell growth	[58]
MMP9	Matrix metalloproteinase 9	Extracellular matrix	[62]
SPARC	Secreted protein, acidic, cysteine-rich (osteonectin)	Extracellular matrix	[62]
TNC	Tenascin C	Cell adhesion	[60, 62]
VEGFA	Vascular endothelial growth factor A	Angiogenesis, vasculogenesis, and endothelial cell growth	[20, 59, 60, 62]
CHI3L1	Chitinase 3-like 1(YKL-40)	Extracellular matrix	[20, 60, 62]
VIM	Vimentin	Cytoskeletal element	[20]

IDH1 mutational status. Here, high expression of *EDNRB* correlates with longer survival whereas the other genes are correlated with higher risk of death. On the basis of the expression of these 4 genes, low-risk and high-risk groups were formed. Interestingly, survival was similar for patients in the low-risk group with wildtype *IDH1* and patients in the high-risk group with mutated *IDH1* [70].

Expression classification and prognosis according to TCGA studies

Studies by The Cancer Genome Atlas (TCGA) have incorporated genomic alterations within expression analyses. Distinct molecular subclasses in high-grade glioma have been identified, delineating a pattern of disease progression that resembles stages in neurogenesis, and have been used to classify glioblastomas into proneural, neural, classic, and mesenchymal subtypes [20, 63, 71]. Proneural glioblastomas are characterized by *IDH1* mutations, and *TP53* and *PDGFRA* alterations, and correlate with a better prognosis and younger age. Classic glioblastomas are differentiated on the basis of high-level amplification of *EGFR*, monosomy of chromosome 10, and deletion of *CDKN2A*. Neural glioblastomas are typified by expression of neuron markers, and resemble normal brain most. Mesenchymal glioblastomas are known for *NF1* deletion or mutation and expression of *YKL-40/CHI3L1* and *MET* [20, 71]. Different subtypes of glioblastoma have been shown to behave differently in response to treatment; Classic and mesenchymal subtypes have a survival advantage after TMZ and RT, whereas the proneural subtype of glioblastomas, with relative good prognostic, does not [71]. Stratified clinical trials in which patient inclusion is based on the genetic alterations that have been identified in their tumor samples are necessary to further increase our understanding of the clinical possibilities of these subgroups.

Epigenetics

Epigenetic silencing mechanisms

Epigenetic silencing of tumor suppressor genes is a common phenomenon of genomic instability in cancer [10]. Epigenetics are inherited characteristics of gene expression, not related to nucleotide sequences. Examples are promoter hypermethylation, histone deacetylation, histone methylation, other histone modifications which can alter chromatin structure (in)directly, and RNA-silencing mechanisms such as RNA interference and microRNA (miRNA or miR) regulation of gene expression [72]. In contrast with the global DNA hypomethylation found in glioblastoma and other tumors [73], tumor suppressor

genes are commonly found to be hypermethylated and, hence, silenced [72]. DNA methylation, histone deacetylation, and miRs are best studied in glioblastoma and are discussed next.

Methylation and histone deacetylation

In glioblastoma, similar to other cancers, global DNA hypomethylation is often seen with hypermethylation of CpG islands in promoter regions. Tumor-suppressor genes frequently found to be silenced by hypermethylation in glioblastoma include *CDKN2A*, *CDKN2B*, *RBI*, *PTEN*, and *TP53*. (reviewed elsewhere [74, 75]). Differences in various genes' promoter methylation have been found between primary and secondary glioblastomas (Table 4) [76–78], long and short-term glioblastoma survivors [75, 79], primary and recurrent tumors, and time to tumor progression [80].

MGMT methylation

Particularly important in glioblastoma is the methylation status of *MGMT*, which is a predictive factor for therapy response and hence survival of glioblastoma patients treated with TMZ and RT [2, 23, 81]. *MGMT* methylation has been observed in 40–57% of glioblastomas; however, specific subgroups have a higher frequency. *MGMT* methylation has been found to be more frequent in secondary glioblastomas [82], in females [83], and in long-term survivors (LTS) [4], whereas it is rare (5%) in recurrent glioblastomas [84]. Conflicting results have been reported regarding the methylation status of *MGMT* as a positive prognostic marker [74, 75, 83]. TMZ and other alkylating agents modify the *O*⁶-position in guanines thereby forming critical DNA lesions that progress to lethal DNA cross-links which prohibit cell replication. The DNA repair enzyme *MGMT* is able to remove alkyl groups, thus introducing resistance to TMZ treatment. However, when the promoter of *MGMT* is methylated, *MGMT* is not transcribed and therefore cannot repair DNA damage caused by TMZ, making TMZ more efficient. The best means of assessment of the *MGMT* methylation status has been debated; the most widely recommended method is methylation-specific PCR (MSP) [85]. Recently, the methylation status of the *FNDC3B*, *TBX3*, *DGKI*, and *FSDI* promoters was identified to be important in patients with *MGMT*-methylated tumors who did not respond to TMZ and RT treatment [79]. *MGMT* methylation is also associated with pseudo-progression after concomitant radiochemotherapy for newly diagnosed glioblastoma patients [86]. Furthermore, the pattern of recurrence, including time to recurrence and location of the recurrent tumor, seems to be correlated with the *MGMT* methylation status of the primary tumor [87].

Table 4 Molecular differences between primary and secondary glioblastoma

Event	Gene symbol	Gene name	Function of encoded protein	Overall frequency in glioblastoma (%)	Frequency in primary glioblastoma (%)	Frequency in secondary glioblastoma (%)	Refs.
Amplification	EGFR	Epidermal growth factor receptor	Regulator of cell signaling, involved in cell proliferation and survival	34	36	8	[18]
Deletion	CDKN2A-P14 ^{ARF}	Cyclin-dependent kinase inhibitor 2A	Regulator of cell cycle	44	44	44	[18]
	CDKN2A-P16 ^{INK4A}	Cyclin-dependent kinase inhibitor 2A	Regulator of cell cycle	26–31	31–32	13–19	[18]
LOH	10q (including PTEN)	Phosphatase and tensin homolog	Regulator of cell signaling, involved in cell proliferation and survival	69	70	63	[30]
	13q (including RB1)	Retinoblastoma 1	Regulator of cell cycle	23	12	38	[30]
	22q (including TIMP3)	TIMP metalloproteinase inhibitor 3	Involved in degradation of the extracellular matrix	53	41	82	[77]
Methylation	19q	?		27	54	6	[30]
	MGMT	O ⁶ -methylguanine-DNA methyltransferase	DNA repair	44	43	73	[83]
	CDKN2A-P14 ^{ARF}	Cyclin-dependent kinase inhibitor 2A	Regulator of cell cycle	14	6	31	[18]
	CDKN2A-P16 ^{INK4A}	Cyclin-dependent kinase inhibitor 2A	Regulator of cell cycle	8	3	19	[18]
	NDRG2	N-myc downstream-regulated gene 2	May have a role in neurite outgrowth	46	62	0	[83]
	PTEN	Phosphatase and tensin homolog	Regulator of cell signaling, involved in cell proliferation and survival	32	9	82	[78]
	RB1	Retinoblastoma 1	Regulator of cell cycle	25	14	43	[76]
	TIMP3	TIMP metalloproteinase inhibitor 3	Involved in degradation of the extracellular matrix	41	28	71	[77]
Mutation	IDH1	Isocitrate dehydrogenase 1 (NADP+)	NADPH production	12–20	4–12	73–88	[9, 39, 41, 42, 44]
	PTEN	Phosphatase and tensin homolog	Regulator of cell signaling, involved in cell proliferation and survival	24–37	25–40	4	[18, 33]
	TP53	Tumor protein p53	Apoptosis	31–38	28–29	65	[18, 33]

A ? indicates that the gene of interest has not yet been identified

Hypermethylation phenotype

A subset of glioblastoma tumors has been found to contain a hypermethylation phenotype at a large number of CpG islands; this has been named the glioma-CpG island methylator phenotype (G-CIMP) by the TCGA. These G-CIMP tumors cluster into the aforementioned proneural subgroup, are strongly associated with *IDH1* mutations, and generally affect younger patients with improved prognosis [88]. Furthermore, inhibition of histone demethylases and TET 5-methylcytosine hydroxylases by mutated *IDH1* potentially implies the methylation of an even greater number of genes in this subgroup [48].

MicroRNAs

miRs are short non-coding RNAs, consisting of approximately 22 nucleotides, which regulate gene expression. miRs usually inhibit target genes' expression, either by inhibiting translation or by triggering the cleavage of the target mRNA. Over 700 miRs have been described in humans [89]. By use of the same methods previously described for gene expression, differences in miR expression have been examined. Compared with normal brain tissue a variety of differentially expressed miRs have been found (Table 5) [90–101].

OncomiRs, tumor suppressor miRs, and therapeutic implications

Frequently up-regulated miRs are called oncomiRs. Of these, miR-26a is found to target PTEN in glioblastomas [102]. Furthermore, miR-26 cooperates with oncogenes *CDK4* and *CENTG1*, forming an oncomiR/oncogene cluster, targeting the RB, PI3K/AKT, and JNK pathways and increasing aggressiveness in glioblastoma [95]. miR-221 and miR-222 are thought to target cell cyclin-dependent kinase inhibitors p27 and p57 by targeting the proapoptotic PUMA [103]. In contrast with these oncomiRs, frequently down-regulated miRs in glioblastoma are considered tumor-suppressor miRs. Of these, miR-7 independently inhibits both the EGFR and AKT pathways [98]. miR-34a suppresses glioblastoma growth by targeting c-Met and Notch [99]. miR-124 and miR-137 target CDK6, which is important in the G1/S-phase transition [97]. miR-128 targets BMI1, which has been shown to promote stem cell renewal [94]. Downregulation of miR-181 is found in responders to temozolomide [100]. The delivery of underexpressed tumor-suppressor miRs may be an appealing approach for therapy. In contrast, overexpressed oncogenic miRNAs may be targeted by antagomirs, because overexpression of the oncomiRs miR-26a, miR-196, and miR-451 has been correlated with poorer survival [93]. A recent

review has provided an up-to-date overview on miRs and their inhibitors for glioblastoma treatment and readers should refer to this for more information [104].

Proteomics

Proteomic studies involve research on the final structure, function, and activity of proteins. Therefore, post-translational modifications on the transcript are included in the results. Thus far, only a limited number of proteomic studies have been performed on glioblastomas and there are still conceptual and technical limitations to overcome [105]. In general, samples are run on 2D gels, which show protein patterns on the basis of size and charge. Proteins identified in tumor samples but not in normal tissue samples are subsequently analyzed by mass spectrometry with matrix-assisted laser desorption/ionization (MALDI) [106]. Thus far, glioma subtypes have been distinguished on the basis of different protein patterns as primary and secondary glioblastomas [107, 108]. Furthermore, on the basis of proteome analysis, survival has been predicted in respect of glioma subtypes [107]. Additionally, proteins' phosphorylation status is a tool with which to identify activated proteins. Consequently, activated receptor tyrosine kinases [109, 110] and the downstream signaling pathways of EGFRvIII have been identified in glioblastomas [111].

Other molecular aspects of glioblastomas

Molecular differences between primary and secondary glioblastomas

Primary and secondary glioblastoma subtypes are histopathologically indistinguishable, but differences can be demonstrated by molecular markers at the epigenetic [77], genetic [1, 18, 24], expression [65], and proteomic [108] levels (Fig. 2; Table 4). Primary glioblastomas have a greater prevalence of EGFR alterations, *MDM2* duplications, *PTEN* mutations, and homozygous deletions of *CDKN2A* [1, 18]. *MET* amplification [24], overexpression of PDGFRA, and mutations in *IDH1* and *TP53* are more prevalent in secondary glioblastomas [1, 9, 18, 33, 39, 41, 43].

The sequential order of molecular alterations

Molecular alterations causing glioblastoma are thought to occur in a sequential order, implicating different stages of gliomagenesis (Fig. 2). For example, *IDH1*-inactivating mutations seem to be an early event in gliomagenesis [43]. In contrast, *PTEN* mutations and LOH 10q are thought to be important in glioma progression, but not initiation [18].

Table 5 Frequently identified microRNA expression alterations in glioblastoma

miRNA	Alteration of expression	Function of encoded protein	Targets	Refs.
miR-7	Decreased	Increases apoptosis, decreases invasion	EGFR	[92, 97, 98]
miR-15	Increased	Regulator of cell-cycle progression	CCNE1	[93]
miR-21	Increased	Oncomir, antiapoptosis	RECK, PDCD4, PTEN	[92, 93, 97]
miR-26	Increased	Induces tumor growth, part of oncomir/oncogene cluster with CDK4 and CENTG1	PTEN and PI3K/Akt pathway	[102]
miR-34	Decreased	Inhibitor of proliferation, survival, migration, and invasion	TP53, c-Met, NOTCH1/2	[99]
miR-124	Decreased	Inhibitor of proliferation, cell differentiation	CDK6, PTBP, SCP1	[97]
miR-125	Increased	Inductor of proliferation and inhibitor of apoptosis	ERBB2, ERBB3, TP53	[92]
miR-128	Decreased	Inhibitor of proliferation	BMI1, E2F3a, EGFR	[92–94]
miR-137	Decreased	Inhibitor of proliferation, cell differentiation	CDK6	[97]
miR-155	Increased	Regulator of immune response in cells	SMAD2	[97]
miR-181	Decreased	Reduced colony formation and migration	TCL1	[92, 100]
miR-196	Increased	Inductor of proliferation, cell differentiation	HOXB8, HMGA2, ANXA1	[93]
miR-210	Increased	Regulator of proliferation	FGFRL-1	[97]
miR-221	Increased	Cell proliferation	p27Kip1, p57Kip2	[90, 92]
miR-222	Increased	Cell proliferation	p27Kip1, p57Kip2	[90]
miR-296	Increased	Inductor of neovascularization	HGS	[91]
miR-326	Decreased	Reduces cell viability and invasion	NOTCH1/2	[101]
miR-451	Increased	Inhibitor of migration, inductor of proliferation	CAB39	[96]

Potential therapeutic targets and future perspectives

Taking into consideration all the molecular alterations found in glioblastomas, it is clear that the picture of the changes in glioblastoma becomes more complex as the techniques that enable us to investigate molecular mechanisms develop. The good news is, however, that many of the alterations identified in glioblastoma cluster in three pathways, the P53 (64–87%), RB (68–78%), and the PI3K/AKT (50%), downstream of the receptor tyrosine kinases (altered 88% in total; Fig. 1). Most alterations occur in a mutually exclusive fashion: alterations within one tumor affect only a single gene in a pathway, suggesting that different genes in a pathway are functionally equivalent [7–9, 71].

Quality of models

Functional validation of the identified molecular changes is essential before they can be assessed as targets for therapy. Taking this into account, it becomes clear that good models are needed for high-throughput testing of rationally designed combinations of drugs with specific targets. Several experiments have shown that established glioblastoma cell lines resemble those of the original glioblastomas very poorly when compared at the level of DNA alterations or gene expression profiles [71]. Tumor neurospheres cultured in stem cell medium, organotypic spheroid cultures, or low-passage monolayer cultures, resemble the

original tumors better and may be better models for study of glioblastomas in vitro [112, 113].

Therapeutic options, multimodal therapy, and delivery options

For optimum application of the insights presented in this paper, stratified clinical trials are necessary to investigate the best treatment options for each common (group of) genetic alteration(s) in glioblastomas. Ultimately, this could lead to more individualized therapies. Rational drug design and rationally designed clinical trials to test these drugs are needed, because an almost infinite number of compounds is currently available, and these can be tested in limitless numbers of combinations. With genomics approaches, discoveries of common features of different types of tumor may lead to new therapeutic targets and drugs for other tumor types also. The discovery of overexpression of VEGFA and its correlation with poor prognosis in glioblastomas [59] led to trials with the angiogenesis inhibitor bevacizumab. It is currently being used to treat recurrent glioblastoma and phase III trials are being conducted [114, 115].

Rather than single-agent therapy, with which good responses have been obtained in the treatment of other types of cancer but which probably will not suffice in the treatment of glioblastoma, combination treatment is necessary. The clinical response of recurrent glioblastomas to EGFR

inhibitors was found, in one study, to be associated with co-expression of EGFRvIII and PTEN [19] or pAkt [116], but not in combination with TMZ and RT for newly diagnosed glioblastomas [117] or in glioblastomas treated with erlotinib and TMZ [118]. PTEN-deficient glioblastoma patients could, for example, be treated with a cocktail of drugs consisting of an EGFR inhibitor and rapamycin [19], however the results are not yet impressive [119]. The response to TMZ and RT of patients for whom *MGMT* methylation is not observed may be improved by addition of *MGMT*-depleting agents, which are currently under investigation [120]. In this respect, the choice of anti-epileptic drug may become important as levetiracetam has been shown to inhibit *MGMT* expression in a preliminary study [121]. In addition, *MGMT*-mediated TMZ resistance may be overcome by more frequent temozolomide doses in dose-dense schedules [122]. Thus far, the results are disappointing, and a putative disadvantage of combination treatment is the potential increase in side effects [123]. This may, in part, be solved by application of new drug-delivery techniques. In this field, advances have been made with the application of biodegradable wafers, convection-enhanced delivery, and strategically-designed liposomes which circumvent the blood–brain barrier [124, 125]. Recent reviews have provided up-to date overviews on therapy, and we refer the reader to those for more details on ongoing and future therapeutic trials [126].

Synopsis

To summarize, our understanding of the molecular mechanisms underlying subgroups of glioblastoma patients has increased. Moreover, many of the alterations in the aforementioned pathways have been elucidated, and molecular typing of glioblastomas on the basis of gene expression has been used to predict prognosis. Furthermore, for the first time it has been shown that the effects of treatment are distinctly different for different molecular types of glioblastoma classified on this basis [71]. In contrast with many other forms of cancer, however, subsequent application of these results to treatment is lagging behind. Nevertheless, assessment of the molecular profiles of responding versus non-responding patients can be used to determine predictive factors and biomarkers, and may lead to identification of new therapeutic targets. Validation of such new therapeutic approaches will be followed by stratified clinical trials based on such molecular subgroups. Finally, current insights will ultimately lead to more individualized therapy for glioblastoma patients. Combination of current knowledge of molecular alterations in glioblastoma with the availability of many drugs with specific targets makes investigation of new treatments more promising than ever before.

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Conflict of interest None.

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